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Confocal imaging to quantify passive transport across biomimetic lipid membranes

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Abstract

The ability of a molecule to pass through the plasma membrane without the aid of any active cellular mechanisms is central to that molecule's pharmaceutical characteristics. Passive transport has been understood in the context of Overton's rule, which states that more lipophilic molecules cross membrane lipid bilayers more readily. Existing techniques for measuring passive transport lack reproducibility and are hampered by the presence of an unstirred layer (USL) that dominates transport across the bilayer. This report describes assays based on spinning-disk confocal microscopy (SDCM) of giant unilamellar vesicles (GUVs) that allow for the detailed investigation of passive transport processes and mechanisms. This approach allows the concentration field to be directly observed, allowing membrane permeability to be determined easily from the transient concentration profile data. A series of molecules of increasing hydrophilicity was constructed and the transport of these molecules into GUVs was observed. The observed permeability trend is consistent with Overton's rule. However, the values measured depart from the simple partition-diffusion proportionality model of passive transport. This technique is easy to implement and has great promise as an approach to measure membrane transport. It is optimally suited to precise quantitative measurements of the dependence of passive transport on membrane properties.

Keywords

Confocal microscope; giant unilamellar vesicle; passive membrane transport

Passive transport through the cell membrane represents a major route by which drugs enter cells. It is the primary route by which orally delivered drugs enter systemic circulation.^{1–2} Environmental toxins can also enter the human body by passively crossing cell membranes.^{3–6} Understanding the mechanistic details of passive transport is essential to understanding how and why certain molecules make good drugs or dangerous toxins, and can further help design suitable drugs.^{7–8}

For over a century, the passive transport of small molecules through lipid bilayers has been understood in the context of Overton's rule, which broadly states that more lipophilic molecules cross bilayers more readily.⁹ More precisely, the consensus holds that membrane permeability is proportional to the product of the molecular diffusivity D and the oil-water partition coefficient K of the permeating species. Since K is expected to vary more than D for small drug-like molecules, oil-water partition coefficients have been widely used to estimate membrane permeability.^{10–12}

Careful mechanistic studies of passive transport are best performed in artificial lipid bilayers, since artificial systems eliminate any active or protein-mediated processes that might be mistaken for passive transport.¹³ Planar lipid bilayers¹⁴ and liposomes^{15–17} are the two most frequently used systems. These approaches are both limited, however. Planar lipid bilayers are physically unstable¹⁸ and contain residual solvent molecules, which introduce artifacts to the permeation results.^{19–21} Studies using the pharmaceutical industry-standard parallel artificial membrane permeability assay (PAMPA), the most common planar bilayer approach, have reported a wide range of permeabilities for drugs such as propranolol and testosterone.²² Another example of the lack of reproducibility in membrane permeation assays was recently presented by Grime and coworkers, who described the wide range of reported permeabilities for weak acids.²³ Techniques used to test liposome permeation include NMR-based and luminescence methods. The NMR-based technique is conducted in steady-state, and cannot yield dynamic information.²⁴ All liposome-based approaches rely on models of liposome permeation that are dependent on the liposome diameter.¹⁶ Liposome polydispersity introduces errors into results from these methods.

One experimental consideration limiting current approaches is the unstirred boundary layer (USL) adjacent to the artificial membrane.²⁵ The concept of the USL originated with stirred planar membrane systems, in which a well-mixed bulk fluid can be maintained on either side of the membrane, but the region directly adjacent to the membrane is stagnant due to non-slip boundary conditions. In this stagnant unstirred region, there is no mixing by convection, and diffusive transport dominates. At steady state, a diffusive gradient is established on either side of the membrane between the bulk concentration and the membrane-adjacent concentration. Of course, the concept of the USL can also be applied to completely unstirred systems; if bulk concentrations are assumed for such a system, Fick's second law results in linear diffusive gradients on either side of the membrane at steady-state.²⁵

In stirred planar membrane systems, the USL extends from 200 μm to 2 mm on either side of the membrane.^{26–27} The USL represents a significant resistance to transport, restricting the permeation coefficients that can be measured.^{23,28} For instance, for a permeability of 10^{-3} cm/s, a 25% measurement error would be introduced by a USL thickness of just 17 μm .²⁵ Thicker USLs result in larger errors. The essence of the USL problem is that while the bulk concentrations on either side of the membrane are easily measured, it is the concentrations directly adjacent to the membrane that determine the membrane flux and that therefore must be known in order to determine the membrane permeability.

We have developed a straightforward solution to the problem of transport artifacts in lipid membrane permeability measurements. Spinning-disk confocal microscopy (SDCM) of giant unilamellar lipid vesicles (GUVs) allows for fluorescent molecules to be tracked as they permeate the lipid bilayer membrane and enter the GUV interior. The speed of spinning-disk confocal microscopy will facilitate the use of this method with fast-permeating molecules. This approach allows for the concentration field to be directly observed, allowing us to establish the complete time course of the evolution of the concentration profile. Precise membrane permeability can be determined easily from the transient concentration profile data by fitting the data to a mathematical permeation model.

In order to investigate how this system reports on the transport of molecules of varying hydrophobicity, we synthesized a series of fluorescently labeled test molecules. This series consists of poly(ethylene glycol) (PEG) with short chains of various lengths attached to nitrobenzoxadiazole (NBD) dyes. We refer to these test molecules as PEG-*n*-NBD, where *n* is the number of ethylene oxide units. This series of molecules provides a well-defined platform for investigating the relationship between hydrophilicity and permeation.

Materials and methods

Materials

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), cholesterol, and biotinylated dipalmitoylphosphoethanolamine (biotin-DPPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Texas Red-modified DPPE (TR-DPPE), succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoate (NBD NHS-ester), and avidin were obtained from Invitrogen (Carlsbad, California). Indium-tin oxide ²⁹-coated glass was obtained from Delta Technologies (Stillwater, MN). Amine-terminated poly(ethylene glycol) alcohols were obtained from Quanta Biodesign (Powell, OH). Poly(dimethylsiloxane) (PDMS) was obtained from Dow chemical corporation (Midland, MI). NoChromix was obtained from Godax Laboratories (Cabin John, MD). All other chemicals were used as provided by Sigma-Aldrich (St. Louis, MO).

Cleaning of glass cover slips

Glass cover slips were first sonicated in water at 80 °C for 30 min, and then dipped in sulfuric acid with NoChromix for 2hr. After rinsing away the acid, the cover slips were sonicated in water again for 30 min, and stored in methanol for later use.

GUV preparation and observation

GUVs were fabricated according to the electroformation technique of Angelova et al.³⁰ from a 1:1:1 (molar ratio) mixture of DPPC, DOPC, and cholesterol. To visualize GUVs, TR-DPPE was added to this mixture at 1 wt% (relative to total lipid mass). To immobilize GUVs on the glass surface, 7 wt% biotin-DPPE was added to the lipid mixture. Lipids were dissolved in chloroform and spread as a thin film on the surface of a piece of ITO-coated glass. After the solvent had evaporated and the remaining film was thoroughly dried, it was rehydrated with a buffer containing 2 mM Hepes at pH 7.0 and 200 mM sucrose. During electroformation, this buffer was heated to 37 °C in a cell formed from two pieces of ITO-coated glass while an oscillating 100 mV signal was applied at 1 Hz.

After 4 h, the GUV suspension was removed from the cell and transferred to a Sykes-Moore chamber constructed with #1 cover glass and containing 200 mM glucose solution. The chamber also contained 0.5–1 μM of the fluorescent test molecule to be transported into the GUVs. The GUVs immediately sank to the bottom of the chamber due to the sucrose-glucose density gradient. There, they were observed by SDCM.

SDCM was performed using a Yokogawa (Tokyo, Japan) CSUX confocal head on a Nikon (Tokyo, Japan) TI-E inverted microscope. Illumination was provided by 50 mW solid-state lasers at 491, 561, or 640 nm. PEG-n-NBD was excited at 491 nm, and emission was captured at 525 nm. TR-DPPE was excited at 561 nm, and emission was captured at 595 nm. An initial image was captured immediately following addition of the vesicles to the buffer containing the fluorescent species. Subsequent images were taken at regular intervals thereafter. Constant illumination, exposure, and camera amplification settings were used across all images in a time series.

Preparation of test molecules

In each synthesis, an amine-terminated poly(ethylene glycol) alcohol containing a well-defined number of ethylene oxide units was reacted with NBD NHS-ester. Reactions were run overnight, at room temperature, in 1:1 (vol:vol) chloroform:methanol with triethylamine added at a 5× molar excess to the amine groups. The reaction products were separated using thin layer chromatography. The reaction scheme is shown in Figure 1, using PEG-4-NBD as an example.

Measurement of octanol-water partition coefficients

1.5 mL of octanol and water were added to a test tube to form a two-phase system. 20 μ L of 1 mM PEG-n-NBD in water was added to the two phase system and the tube was vortexed until the solution within appeared uniform. The two phases were allowed to settle for 10 min. Then UV-visible absorption spectrum of PEG-n-NBD in each phase was measured using a Shimadzu (Kyoto, Japan) Biospec-1601 spectrometer at 465 nm. The extinction coefficients ϵ of NBD at 465 nm in water and octanol are 24000/M/cm and 19000/M/cm³¹ respectively. The concentration of PEG-n-NBD in each phase was calculated from ϵ according to the Beer-Lambert law. Partition coefficients were calculated as $K_{oct-water} = C_{oct}/C_{water}$.

Buffer exchange on a single GUV

For experiments investigating a single GUV with different chemicals present in the exterior space, GUVs were immobilized on the #1 cover glass surface of a microfluidic channel. The channels were made from PDMS patterned via standard polymer micromolding techniques.³² The patterned PDMS and a #1 cover slip were oxidized by corona treatment (BD-20AC, Electro-Technic Products, Chicago, IL) to bond them irreversibly.³³ The PDMS channel was a T-junction³⁴ configuration with two inlets and one outlet. The channel used to observe GUVs had a width of 1 mm, depth of 100 μ m, and length of 1 cm. Buffer solutions were injected into either inlet using a Harvard Apparatus (Holliston, MA) syringe pump. The linear flow velocity in the observation channel was 5 mm/sec.

To immobilize GUVs on the glass surface, the biotin-avidin interaction was used. GUVs were fabricated with biotin-DPPE in the lipid mixture as described above. To functionalize the surface with avidin, a 25 μ g/mL avidin solution was pumped into the device and incubated for 10 minutes at room temperature. Afterwards, the surface was gently flushed with glucose buffer for 1 min to remove unbound avidin. Then, the functionalized surfaces were incubated with vesicles for 20 min. Unbound vesicles were removed by gently washing the sample channel with buffer solution for 1 min.

To observe background fluorescence, 0.1 mg/mL of 40 kDa fluorescein-dextran in glucose buffer (200 mM glucose, 2 mM HEPES, pH 7.0) was first added to the observation chamber and images were captured. 1 μ M PEG-NBD solution was flowed into the channel to flush away the fluorescein-dextran solution.

Image analysis

Images were analyzed using ImageJ (freely available from the NIH; rsbweb.nih.gov/ij/) and Matlab (The MathWorks, Inc., Natick, MA). To correct for illumination heterogeneity, the images were flat-fielded in reference to an image of an empty (no GUVs) field at the same fluorophore concentration. Pixel-by-pixel multiplicative factors necessary to make the blank field uniform were thereby derived; this array of multiplicative factors was then applied to the data images.

Data processing

For each vesicle permeation experiment, the solution concentration of PEG-n-NBD ($n=4, 8,$ and 12) and camera settings were the same. The total fluorescence intensity of a 2–5 μ m diameter circle was measured at the center of the GUV. This measurement was used as the internal intensity. The intensity was also measured in 10 circles with the same diameter evenly distributed outside of the GUV; deviation from the mean of these measurements was propagated as a measurement error throughout this analysis. The mean of the exterior measurements was used as the nominal exterior intensity. To correct for photobleaching and laser drift, the external fluorophore concentration was assumed to be constant (the vesicle

volume is negligible compared to the total volume). Based on this assumption, internal and external intensities were adjusted by the same additive factor necessary to maintain corrected external intensity constant throughout the experiment. These values were then baseline-subtracted using the initial internal intensity.

Results and discussion

Confocal Imaging of Membrane Permeation

An initial image was captured immediately following addition of GUVs. Subsequent images were taken at regular intervals thereafter. A typical time series of images is shown in Figure 2. This time series shows the change in internal fluorescent intensity as PEG-8-NBD permeates the lipid bilayer of a GUV. The PEG-8-NBD concentration can be seen to gradually increase inside the GUV until the internal and external intensities match. This indicates that rather than reaching a non-equilibrium steady state, the system achieves diffusive equilibrium across the membrane.

Concentration Profile

To observe the concentration profile adjacent to the vesicle membrane and characterize any USL, line intensity profiles through vesicles were measured. The trace labeled PEG-NBD in Figure 3a is an example of such a profile. These data are from an experiment with PEG-4-NBD penetrating the vesicle taken at the 1 min time point. The gradual decrease in fluorescent intensity between the membrane and the vesicle center is an artifact of SDCM imaging. Confocal microscopy does not perfectly reject light from outside of the focal plane. In the regions at the edge of the vesicle, where the distance through the vesicle along the imaging axis is smaller, more light from fluorophores external to the vesicle leaks through.

To correct for this effect, we used a buffer exchange system with a fluorescent molecule that cannot penetrate the membrane. The non-penetrating fluorophore we selected was 40 kDa fluorescein-dextran. The fluorescein-dextran trace in Figure 3a (labeled FI-dextran) is a line profile with fluorescein-dextran exterior to the vesicle. Notice the resemblance of the curve interior to the membrane to the trace labeled PEG-NBD. To correct for out-of-plane light leakage, the control fluorescein-dextran trace in Figure 3a was subtracted from the PEG-NBD trace in 3a. The result, shown in Figure 3b, is a flat intensity profile throughout the vesicle interior. It is clear that the concentration rapidly goes to the bulk concentration on either side of the membrane. As estimated from the diffusion profiles of Figure 3, the length of the USL interior to the GUV membrane can be no more than 1 μm ; it is 5 μm or less exterior to the vesicle.

The curved profiles shown in Figure 3a are consistent with theoretical expectations of out-of-plane light leakage in SDCM. According to Sandison and Webb,³⁵ the image fluorescence from a single out-of-focus spot is given by:

$$I_b[v_l, u] \propto \left(\frac{16}{u^4}\right) 2 \left[u^2 \cos^{-1} \left[\frac{v_l}{2u} \right] - \frac{v_l}{2} \left(u^2 - \frac{v_l^2}{4} \right)^{1/2} \right] \quad 1$$

where v is the distance from the optic axis, and u is the distance from the in-focus plane. The fluorescence leakage into an image plane inside the vesicle can therefore be obtained by integrating the fluorescence from outside the vesicle. To perform this integration, we treated the vesicle as a sphere, with the edges of the sphere closer to background fluorophores than the center of the sphere. Figure 4 is a comparison of the fluorescence leakage along the vesicle axis calculated by theory and intensity data from the fluorescein-dextran experiment,

demonstrating that the intensity profile observed can be explained by out-of-plane fluorescence in a spherical geometry.

Correcting for GUV size-dependence of the fluorescence background

As described above, since SDCM does not perfectly exclude out-of-focal-plane light, there is some fluorescence background present in the vesicle interior in each image. Since the amount of light leaking into the focal plane is greater for fluorophores closer to the focal plane, the background signal is greater for smaller vesicles (in which the “bulk” fluorescence region is closer to the image plane). To analyze data from GUVs of various sizes, we had to correct for this effect. Figure 5 shows intensity at the center of vesicles incubated with 40 kDa fluorescein-dextran as a function of vesicle diameter. This relationship, captured by the empirical polymeric fit of equation 2, was used to compare vesicles of various sizes. The center vesicle intensities were adjusted to the intensity expected for a reference diameter of 40 μm .

$$I = 1.5d^2 - 200d + 21000 \quad 2$$

Permeability Calculations

Permeabilities were calculated from corrected, baseline-subtracted vesicle internal intensities. As the fluorescent intensity is proportional to concentration, the intensity was used as a proxy for concentration. To obtain permeability values, we utilized a simple model of the temporal development of the concentration field. Molecular flux across the membrane was treated as a simple permeation process: $J = P(c_o - c_i)$, with flux J , permeability P , and concentrations c outside and inside the vesicle. External concentration c_o was treated as a constant; internal concentrations c_i were normalized by this constant. For a single vesicle, $J = N' / A$, where N' is the first time derivative of number of molecules and A is the surface area of the vesicle. The concentration inside the vesicle is simply $c_i = N/V$, where V is the volume of the vesicle. Substituting into the permeation equation yields a first order ordinary differential equation:

$$N' + \frac{AP}{V}N = APc_o \quad 3$$

If we apply the boundary condition that at $t=0$, $c_i=0$ and the vesicle is spherical with diameter d , the solution to this equation is:

$$c_i(t) = c_o \left(1 - e^{-\frac{6P}{d}t} \right). \quad 4$$

We obtained temporal fluorescent intensity profiles for PEG-*n*-NBD molecules with $n = 4, 8$, and 12. Representative results are shown in Figure 6, along with a single-parameter (P) fit to equation 4. For each species, the time course of permeation into three vesicles was observed. The average permeabilities obtained were: PEG-4-NBD: $1.13 \pm 0.08 \times 10^{-5}$ cm/s; PEG-8-NBD: $2.04 \pm 0.17 \times 10^{-7}$ cm/s; PEG-12-NBD: $2.27 \pm 0.21 \times 10^{-8}$ cm/s. Measured permeability is independent of vesicle diameter.

To compare this method to previous investigations, we used the same procedure to measure the permeability of fluorescein at pH 7.0 as $1.94 \pm 0.18 \times 10^{-5}$ cm/s. This is within the range measured by other researchers: from $1.6 - 21.2 \times 10^{-6}$ cm/s in a pH range from 5.6 to 8.06, with maximum permeability observed at pH 6.5 and below.^{36–38}

The potential effect of the unstirred layer was calculated based on the observations shown in Figure 3 according to the equation described by Barry and Diamond²⁵:

$$1/P = (1/P_m) + (\delta'/D') + (\delta''/D'') \quad 5$$

Here P is the apparent permeability, P_m is the actual membrane permeability, δ' is the USL thickness inside the vesicle, δ'' is the USL thickness outside the vesicle, and D is the diffusivity inside the unstirred layer. In our experiment, the unstirred layer thicknesses inside and outside the vesicle were no more than 1 μm and 5 μm respectively (see Figure 3b). According to Graham's law, for small molecules, the square root of molecular weight and diffusivity are inversely proportional.³⁹ Based on the measured diffusivity of fluorescein-biotin⁴⁰, which has a molecular weight close to that of PED-12-NBD, we can estimate the diffusivities of PEG-4, 8, and 12-NBD as 5.1, 4.2, and $3.6 \times 10^{-6} \text{ cm}^2/\text{s}$ respectively. If P is the permeability obtained from the experiments, P_m can be derived from equation 5. The percent error introduced by the USL can be used to estimate the effects of USLs in these experiments. For PEG-4-NBD, this value is no more than 0.1%. The value for PEG-8-NBD is $2 \times 10^{-3}\%$, and for PEG-12-NBD it is $2 \times 10^{-4}\%$.

The octanol/water partition coefficients $K_{\text{oct/water}}$ of the three test molecules, listed according to increasing PEG chain length, were measured as 2.88 ± 0.24 , 0.45 ± 0.05 , and 0.20 ± 0.02 . In other words, as lipophilicity of the molecules decreased, permeability decreased as well. This result is consistent with several recent results showing that more lipophilic molecules have larger permeabilities. For instance, Walter and Gutknecht tested the permeability of egg phosphatidylcholine-decane planar bilayer membranes to weak carboxylic acids with alkane tails ranging from formic to hexanoic acid. Carbon isotopes were used as tracer to track the acid concentration change. They found that as the hydrocarbon chain length increased (the acids became more lipophilic), the permeation rate increased, which is consistent with Overton's rule.⁴¹ Pitkanen and coworkers tested the permeability of a retinal pigment epithelium (RPE) to β -blockers exhibiting a wide range of lipophilicity, determining permeant concentration by UV absorbance. They found that the permeabilities of lipophilic β -blockers were up to 20 times higher than those of hydrophilic molecules.⁴²

The permeation coefficient P is widely described by Overton's rule in terms of the oil-water partition coefficient K , as $P = KD_{\text{mem}}/l$, where D_{mem} is the diffusion coefficient of the molecule in the membrane and l is the membrane thickness.⁴³ Generally $D_{\text{mem}} = D_0 M^{-1.22}$, where D_0 is the calculated diffusion coefficient for a solute of unit molecular weight.⁴⁴ Substituting K and D_{mem} into the Overton equation for permeability, the predicted permeabilities of these three molecules have a ratio of 32:3:1 (see Table 1). But in the permeation experiments, P changed nearly an order of magnitude more than this, indicating that there are factors other than diffusivity and oil-water partition affecting membrane permeability. Examples of such factors were recently explored by Xia and coworkers, who used Abraham's linear solvation energy relationship (LSER) to predict solvent effects on membrane absorption, taking into account lone-pair electrons, effective dipolarity and polarizability, and hydrogen bonding.⁴⁵ A careful analysis of molecular properties in addition to lipophilicity is likely to result in more accurate predictions of membrane permeability.

Conclusions

We have developed a straightforward solution to the problem of artifacts in lipid membrane permeability measurements. We use confocal microscopy to image the transport of

fluorescent molecules into GUVs. Confocal microscopy allows for the interior of GUVs to be trivially distinguished from their exterior. Fluorescent molecules in the exterior space can therefore be optically tracked as they permeate the membrane and enter the GUV interior. Since this technique is easy to implement, it has great promise as an approach to measure membrane transport properties. It can be generalized to a variety of transported molecules by using fluorescence systems based on, for example, pH-sensitive fluorophores or fluorogenic molecular reporters. It is optimally suited to precise quantitative measurements of the dependence of passive transport on membrane properties, including lipid bilayer composition, charge state, curvature, and phase. These are central issues in understanding how molecules passively diffuse into cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Preparation of the PEG-4-NBD test molecule. An amine-terminated 4-unit poly(ethylene oxide) alcohol was reacted with an NHS-ester of the fluorescent dye NBD. To prepare other PEG-n-NBD molecules, PEG precursors with different numbers of repeating units were used.

Figure 2.

a. A time series of SDCM images showing the transport of the fluorescent molecule PEG-8-NBD into a GUV. b. GUV labeled by Texas-Red-modified DPPE showing the lipid bilayer. Scale bars are 10 μm.

Figure 3.

Intensity profiles drawn through the center of the GUV. a) A GUV with PEG-4-NBD permeating (blue trace), and the same GUV with fluorescein-dextran (40kD) outside (red trace), b) The PEG-4-NBD line profile through the vesicle corrected to remove background fluorescence.

Figure 4. Comparison of the fluorescence profile calculated by out-of-plane fluorescence theory with experimental data obtained from a GUV with 40 kDa fluorescein-dextran outside.

Figure 5.
Dependence of background intensity at the center of GUVs on GUV diameter.

Figure 6. Plot of fluorescence intensity inside over intensity outside GUVs (1:1:1 DPPC:DOPC:cholesterol) as a function of time at 26 °C. Data points are red circles with black error bars; regressions of P in Equation 4 to the data are blue lines.

Table 1

Comparison of Overton's rule to experimental results. Values were normalized to PEG-12-NBD

	$K_{oct/water}$	$D = D_0 M^{1.22}$	$P_{calc} = KD/l$	P_{exp}
PEG-4-NBD	14.4	2.2	32	497
PEG-8-NBD	2.3	1.4	3	9
PEG-12-NBD	1	1	1	1